THE INITIATION OF DEVELOPMENT IN CHÆTOPTERUS.

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I. Introduction.

The object of the experimentation described in this paper was a partial analysis of the initiation of development in the egg of the marine annelid, *Chætopterus pergamentaceus*. The experiments were made and material collected at the Marine Biological Laboratory in Wood's Hole during the summers of 1909, '10 and '11.

Two methods of attacking the problem were employed,—the first, that of inducing artificial parthenogenesis,—the second, that of combining fertilization and artificial parthenogenesis. No investigator has yet succeeded in inducing normal development by parthenogenesis in *Chætopterus*, although varying degrees of approximation to this result have been attained. A variety of methods, which have proved more or less effective in other forms, have therefore been employed, in the hope that such experiments might throw light on certain of the factors concerned in the initiation of development in this particular form.

A. Normal Development.—The egg differs from many eggs in having a membrane present before fertilization. About ten minutes after the entrance of the spermatozoan the first polar body is extruded, and ten minutes later the second. The first cleavage takes place forty minutes after fertilization, and is preceded by the formation of a large polar lobe, the contents of which passes into one of the two daughter cells. The first cleavage is thus unequal. The history of the cleavage is essentially like that of other annelids. The blastula becomes ciliated, and swims at six or seven hours. The larval form is a trochophore, uniformly ciliated, with a long apical tuft of cilia at the anterior end. Among any set of normal, fertilized eggs, there are very likely to be found varying numbers of unsegmented, ciliated, swimming larvæ. Cleavage is thus seen to be unnecessary to a certain amount of differentiation and development. This was shown experimentally by F. R. Lillie (1902). These larvæ do not develop the typical form nor the apical tuft of cilia.

B. Artificial Parthenogenesis.—In artificial parthenogenesis the development follows much the same course as in fertilization, but the experiments enable us to resolve the development into a separable series of steps. The first step is the formation of the

metaphase of the first maturation spindle, the second the extrusion of the first polar body, the third the completion of maturation, the fourth is cleavage (the second, third and fourth may be omitted), the fifth shows varying degrees of differentiation up to the formation of swimming larvæ of greater or less activity. A graded series of stimuli may be determined in which the development may be made to stop at almost any step in the process.

The chief difficulty in the artificial parthenogenesis has been to induce cleavage. Formerly all successful efforts to induce parthenogenesis in *Chætopterus* resulted in the production of unsegmented larvæ only.[1] One of my principal objects has been to discover, if possible, something of the conditions determining cleavage. I have found many agents that will induce the development of swimming larvæ without cleavage, but only two thus far which led to the production of segmented larvæ,—(1) subjecting the eggs to the action of heat, and (2) the use of oxygen-saturated sea-water after a short exposure of the eggs to a potassium chloride solution. The latter gave segmented larvæ in so few cases that I consider the results as somewhat uncertain. Of the heat results there is no doubt. The fact that so many fertilized eggs develop without cleavage suggests that any abnormal condition is likely to have as a result the suppression of cleavage,—that the *balance* of developmental phenomena is a delicate one. The variety of agents that will induce development of some sort indicates that the egg is in a rather easily disturbed equilibrium,—a very *labile* condition.

[1] Since sending this paper to the BULLETIN I have read the account by Professor Jacques Loeb and Dr. Hardolph Wasteneys, "Fertilization of the Eggs of Various Invertebrates by Ox-Serum," published in *Science*, Vol. 36, No. 921, 1912. In this paper they announce the production of cleavage in unfertilized eggs of *Chætopterus* by the following method. "We induced segmentation in the eggs of *Chætopterus* by putting them for from 1½ to 2½ minutes in a mixture of 25 c.c. ⅜ *M* strontium chloride + 25 c.c. *M*/2 NaCl + CaCl + KCl, then for ten minutes into ox-serum diluted with its own volume of the above mentioned solution and then by putting them for thirty minutes into hypertonic sea-water." The parthenogenetic agent is considered to be the lysin in the ox-serum, and the authors' conclusion is for *Chætopterus* as for the sea-urchin, that the first phase of fertilization consists in a superficial cytolysis.

TABLE I.

AGENTS INDUCING THE FORMATION OF SWIMMING LARVÆ UNSEGMENTED EXCEPT IN THE CASE OF HEAT, OR OF KCl FOLLOWED BY OXYGEN EXCESS IN THE SEA-WATER.

Potassium chloride, 2½ *M* in sea-water.
- 0.5 per cent.—10½ minutes to 5 hours and 10 minutes.
- 1.0 per cent.—3 minutes to 5 hours and 10 minutes.
- 2.0 per cent.—21 minutes to 3 hours and 10 minutes.
- 3.0 per cent.—45 and 60 minutes. (Shorter exposures not made.)
- 3.5 per cent.—1 minute to 3 hours.
- 4.0 per cent.—45 to 60 minutes. (Other exposures not made.)
- 5.0 per cent.—1 minute to 3 hours and 10 minutes.
- 6.0 per cent.—45 and 60 minutes. (Other exposures not made.)
- 7.5 per cent.—5 minutes to 4 hours.
- 10.0 per cent.—5 minutes to 3 hours and 58 minutes.
- 15.0 per cent.—1 minute to 31 minutes.

n/10 acetic acid in sea-water.
- 1.0 per cent.—10½ minutes.
- 5.0 per cent.—5 minutes.

Low temperature.
- 9.5° C.—1 hour or 2 hours and 17 minutes.
- 12.5° C.—2 hours and 17 minutes.

Oxygen-saturated sea-water.
- 1 hour to 2 hours.

De-aerated sea-water (after 2 per cent. 2½ *M* KCl 3 minutes)—over night.

De-aerated sea-water (with 2 per cent. 2½ *M* KCl)—1 hour and 30 minutes.

Alcohol in sea-water.
- 5 per cent. 40 minutes.

High temperature.
- 33°–34° C.—25 minutes to 1 hour and 30 minutes.
- 32.5°–34.5°—40 minutes to 1 hour and 30 minutes.
- 30°–30.5°—40 minutes.
- 33.5°–34.5°—25 minutes to 40 minutes.

Sodium chloride, 2½ *M* in sea-water.
- 13.7 per cent.—5 minutes and 1 hour and 10 minutes.

2½ *M* sodium chloride and *n*/10 sodium hydroxide, in sea-water.
- 8.0 c.c. NaCl + 50 c.c. sea-water + 0.2 c.c. NaOH—20 minutes to 18 hours.
- 8.0 c.c. NaCl + 50 c.c. sea-water + 0.4 c.c. NaOH—20 minutes to 18 hours.
- 8.0 c.c. NaCl + 50 c.c. sea-water + 1.0 c.c. NaOH—50 minutes to 18 hours.

n/10 hydrochloric acid in sea-water.
- 2 per cent. permanently.

Fatty acid followed by hypertonic sea-water.

n/10 butyric acid 2.8 c.c. + 50 c.c. sea-water—2 minutes; then 200 c.c. sea-water 20 minutes; then 8.0 c.c. 2½ *M* NaCl + 50 c.c. sea-water 30 minutes.

Potassium chloride followed by hydrogen current through sea-water.
- 3.5 per cent. 2½ *M* KCl in sea-water—45 or 65 minutes, then hydrogen 3 hours.

Potassium chloride followed by excess of oxygen in sea-water.
- 3.5 per cent. 2½ *M* KCl in sea-water until after formation of second polar body, then oxygen saturated sea-water 30 min. to 4 hours.

TABLE II.

AGENTS INDUCING POLAR BODY FORMATION, BUT NO SWIMMING LARVÆ.

$2\frac{1}{2}$ *M* potassium chloride in sea-water.
0.1 per cent.—1 minute to 25 minutes.
0.5 per cent.—5½ minutes, sometimes 10 or 20 minutes.
1.0 per cent.—1 minute, sometimes 3–6 minutes.
2.0 per cent.—1 minute to 10 minutes.
3.5 per cent.—4 minutes (in some cases).
5.0 per cent.—4 minutes (in some cases).
7.5 per cent.—1 minute.
10.0 per cent.—1 minute.
15.0 per cent.—5 minutes (in some cases).
n/10 acetic acid in sea-water.
1.0 per cent.—1 or 5 minutes.
Low temperature.
9.5° C.—1 hour (in some cases).
12.5° C.—15 minutes.
De-aerated sea-water.
10 minutes to 1 hour and 16 minutes.
Oxygen-saturated sea-water.
16 minutes to 1 hour and 5 minutes.
n/10 potassium cyanide in sea-water.
20.0 per cent.
Double potassium chloride, in some cases.

TABLE III.

AGENTS INEFFECTIVE IN INDUCING DEVELOPMENT.

Potassium chloride, $2\frac{1}{2}$ *M* in sea-water.
1.0 per cent.—1 minute (in some cases).
7.5 per cent.—5 hours and 30 minutes.
10.0 per cent.—5 hours and 30 minutes.
15.0 per cent.—1 hour 55 minutes to 5 hours 30 minutes.
Mechanical agitation or nicking.
Low temperature.
9.5° C.—15 or 30 minutes.
Sperm extract, by boiling.
Alcohol in sea-water.
0.5 per cent.—3 minutes to 3 hours 15 minutes.
2.0 per cent.—3 minutes to 3 hours 15 minutes.
5.0 per cent.—3 minutes to 3 hours 15 minutes (except 40 minutes).
Oxygen-saturated sea-water.
10 or 20 minutes (in some cases).
High temperature.
30°–31.3° C.—5 minutes or 1 hour 30 minutes.
33°–34.2° C.—5 minutes or 20 minutes.
35°–45° C.—5 minutes to 60 minutes (except perhaps 40°,—1 minute).
Nitric acid.
Sulphuric acid.
Oxalic acid.

Tables I.–III. show a list of the agents employed, with the varying degrees of success. As stated in Table I. swimming larvæ may be induced in unfertilized eggs by the use of potassium chloride, sodium chloride, hydrochloric acid, acetic acid, butyric acid followed by sodium chloride, sodium hydroxide and sodium chloride, alcohol, potassium chloride followed by hydrogen or by oxygen-saturated sea-water, or by de-aerated sea-water, low-temperature, high-temperature, or oxygen-saturated sea-water. Probably there is a large number of other agents also which would bring about the same result. The majority of the above may be made to induce polar body formation only, if shorter time or lower concentration is used (see Table II.). Potassium cyanide, and de-aerated sea-water, both induced polar bodies only, no matter what time or concentration was used. The same agents may prove altogether ineffective in inducing development when used in certain other concentrations and times. Sulphuric, nitric, and oxalic acids, were also found to be ineffective, as well as sperm extract (by boiling), and mechanical agitation and injury (by shaking with ground glass) (Table III.). Many of the experiments were performed merely to obtain a "lead," and if the results did not appear suggestive that line of work was abandoned. If they appeared suggestive the experiment was usually repeated with some changes.

Considerable variation was noted in the behavior of different lots of eggs under similar conditions of experimentation. Just what the conditions are which govern this variation I have not been able to discover. Sometimes the female did not appear to be in good condition, but this was not a universal test by any means. Variations in results under uniform conditions seem to be characteristic of experiments in artificial parthenogenesis.

C. Methods.—The animals were brought to the laboratory in their tubes. On removal from the tubes, the males and females were kept in separate dishes in running sea-water, from twelve hours upward. In some cases the females were washed in tap water for one minute, before using, but this did not seem to be a necessary precaution. On using, the sex segments were cut off, the ovaries removed, and torn to pieces, and the eggs passed

through cheese-cloth to free them from body fragments and from some of the mucus, which is very thick.[1]

The eggs were left in sea-water, after removing from the ovaries, from twenty to forty minutes, in order that the first maturation spindle might form before further treatment. Experiments showed that eggs so treated formed a larger percentage of second polar bodies. Such eggs formed the polar bodies more quickly, both in normal fertilization and in experiments. Unfertilized controls were always run, and fertilized controls also if the experiment included the effect of the combination of fertilization and artificial parthenogenesis. Care was taken, of instruments and dishes, to keep all unfertilized eggs free from contamination from sperm.

A considerable amount of material was fixed for cytological study, largely covering the ground of the experiments. The study of the fixed material was used to amplify and check that of the living eggs. The eggs used for this work were killed either in Boveri's picro-acetic; in Flemming's (weak) solution; or in Meves's fluid. They were stained in Heidenhain's iron-hæmatoxylin or in thionin,—and counter-stained in Orange G. The sections were cut four micra thick.

II. The First Changes in Development.

So long as the eggs remain in the ovary the germinal vesicle is intact, but almost immediately upon entrance into sea-water the vesicle breaks down, its contents migrate to the animal pole, and the metaphase of the first maturation spindle forms. At the same time the membrane, which could be seen from the beginning, stands off from the egg at a greater distance, forming a distinct space between the membrane and the egg surface. Here development pauses, and unless further stimulus is given the eggs remain in this condition, and after a number of hours go to pieces.

The question of determining the factors which bring about these first developmental changes is a problem of which I have had

[1] It is probable that one reason so many of the controls died before the close of the experiment was that more mucus was left in the control dishes than in the ones in which the solutions were changed a greater number of times.

opportunity to make only the most hasty examination. It might well repay further work. The method employed was to open up the ovaries in solutions other than sea-water: The female was rinsed one minute in distilled water, to wash off the sea-water, then dried on filter paper sufficiently to remove external moisture. The sex segments were then cut off and placed in the desired solutions, where the eggs were teased out. The results were as follows:

A. Body Fluid.—When no solution was supplied and the eggs were merely allowed to come out of the ovary into a drop of body fluid (which is plentiful in the sex segments outside the ovary tubes), the germinal vesicle broke down, its contents migrated to the animal pole, the spindle formed, and the membrane assumed its normal relation to the egg. In short the whole process appeared to be normal. Since the body fluid can produce these changes it must be supposed either that the ovarian wall is impermeable to the body fluid, or that an oxygen supply greater than that within the ovary must be furnished in order to induce the changes. To test this last suggestion sex segments were opened up in water which had been de-aerated by boiling.

B. De-aerated Sea-water.—In this case the female had not been dried as formerly, since sea-water was to be used. Eggs taken from the ovary in de-aerated sea-water behaved normally in all respects. It is of course possible that sufficient oxygen was carried in with the eggs to have an effect on the processes, but it scarcely seems probable that much increase in oxygen supply over that present in the ovaries was furnished the eggs in the experiment. Therefore, it would appear that the reason that the eggs do not go through the first stage in the ovaries is that the wall of the ovary is semi-permeable to the body fluid, not allowing a sufficient amount of it to enter to cause the eggs to go through the changes described.

C. Salt Solutions.—In order to determine whether any of the salts in the sea-water were necessary factors in the determination of these first developmental changes, solutions of various salts were used,—$n/2$ sodium chloride, $n/2$ potassium chloride, $n/2$ calcium chloride, and $n/2$ magnesium chloride. The $n/2$ sodium chloride and $n/2$ potassium chloride led to the break-down of the

germinal vesicle, the migration of its contents to the animal pole, and the formation of an apparently normal spindle, but not to normal membrane relations. The *n*/2 calcium chloride and *n*/2 magnesium chloride led to the break-down of the germinal vesicle, but the migration of the contents of the vesicle to the animal pole did not follow. In the *n*/2 magnesium chloride the membrane relations were fairly normal,—but not so in the *n*/2 calcium chloride.

Combinations of the salts were made according to the following Van't Hoff formula:

$\frac{n}{2}$ NaCl—10 c.c.

$\frac{n}{2}$ KCl—0.22 c.c.

$\frac{n}{2}$ $MgCl_2$—1.2 c.c.

$\frac{n}{2}$ $CaCl_2$—0.2 c.c.

When 10 c.c. of *n*/2 NaCl was used with 1.2 c.c. of *n*/2 $MgCl_2$ (no KCl or $CaCl_2$ being present), the results were normal in all particulars. Evidently then, potassium chloride and calcium chloride are unnecessary to the first developmental stage. When, however, 0.22 c.c. of *n*/2 KCl or 0.2 c.c. of *n*/2 $CaCl_2$ were substituted for *n*/2 $MgCl_2$ with *n*/2 NaCl, the spindles formed as normally but the membrane relations were not normal. Magnesium chloride seems to have some definite effect in inducing normal membranes, therefore, and either *n*/2 sodium chloride or *n*/2 potassium chloride may induce normal spindles. Salt solutions are not necessary to induce certain of the developmental changes, however, as is seen by experiments with neutral paraffin oil and with cane sugar.

D. Neutral Paraffin Oil.—When the ovaries were opened in this liquid a certain number of eggs, although not all by any means, appeared to behave normally in that the vesicle contents migrated to the animal pole, and the maturation spindle formed,—but the eggs swelled slightly and the membrane did not stand off from

the egg as normally. The result may be complicated by the fact that a small amount of body fluid is always present, and does not mix with the oil. However, where the eggs seemed to be comparatively free from body fluid in the oil, the results were as stated. It is possible that the space between the membrane and the egg may be obliterated by the swelling of the egg.

E. Cane Sugar Solutions.—*n*, 5/8*n*, and *n*/2 solutions of cane sugar were tried. All of the solutions injured the eggs considerably. In the 5/8*n* sugar the membrane swelled away from the egg, leaving a space much larger than normal, while the egg itself appeared to be plasmolyzed. Evidently fluid had been extracted from the egg into the vitelline space by the injurious action of the sugar solution. In a few eggs in all the solutions the germinal vesicle broke down, its contents migrated to the animal pole, and something resembling an abnormal polar spindle was formed.

The experiments are too few to draw from them definitive conclusions. It seems possible, however, that the internal condition of the egg is a sufficient stimulus to cause the first developmental stage, provided that conditions be furnished which are favorable to the reactions involved, that is, that the egg needs no stimulus from without, and that the failure of the germinal vesicle to break down in the ovary is due merely to an inhibition of the processes because of a lack of favorable conditions in the ovary,—that all that is necessary is a release of the process in the egg.

III. Study of Artificial Parthenogenesis in Chætopterus by Means of Single Agents.

A. The Action of Salt Solutions. 1. *Potassium Chloride.*—Mead ('98), Loeb ('01) and F. R. Lillie ('02) used potassium chloride to induce development in *Chætopterus*,—Mead only carrying the eggs through maturation and a few of the earlier developmental changes, Loeb performing a number of experiments to determine the nature of the KCl effect, and Lillie making an extensive study of the course of the development, with living and fixed material. Following their lead I have used potassium chloride as one of my principal agents. The cytological results of potassium chloride treatment are very interesting and appear fairly typical of

development without cleavage, induced in any way whatever,—by parthenogenetic agent or by sperm. There is space here for a general account only, but I hope to give a more extended treatment of the subject at another time. The hope was entertained for some time that the right concentration or time of application of potassium chloride might bring about cleavage, but the method has never been successful for that purpose.

In the experiments a certain number of cubic centimeters of 2½ *M* KCl were mixed with a given amount of sea-water. The eggs were placed in this solution after remaining 20–40 minutes in ordinary sea-water (a delay which hastened and aided development),—and later were returned to normal sea-water after a given exposure to the action of the KCl.

A. Experiments to Determine the most Favorable Concentration and Time for Development.—Numerous experiments were performed to determine the best concentration, and time of KCl exposure to bring about the largest percentage of development. Tables IV. and V. are taken from this set of experiments. By comparing the tables it will be noted that there is a considerable difference between the concentration and time inducing the best percentage of maturation and that inducing the greatest production of swimmers. Brief exposures give the best results for maturation, but the greatest number of swimmers arises from longer exposures. The best percentage of maturation,—100 per cent. of eggs completing maturation,—was gained by treatment

TABLE IV.

TO SHOW THE BEST LENGTH OF EXPOSURE TO A GIVEN POTASSIUM CHLORIDE SOLUTION TO INDUCE THE FORMATION OF BOTH POLAR BODIES.

Concentration of 2½ *M* KCl, in Sea-water, Per Cent.	Time of Exposure.	Per Cent. Forming Polar Bodies.
0.1	9.5 minutes	14
0.3	10.5 minutes	29
0.5	10.5 minutes	61
1.0	3–6 minutes	100
2.0	3–5 minutes	81
3.5	1+minute	100
5.0	1 minute	59
7.5	1 minute	80
10.0	1 minute	41
15.0	1 minute	64.5

TABLE V.

TO SHOW THE BEST LENGTH OF EXPOSURE TO A GIVEN SOLUTION OF POTASSIUM CHLORIDE TO INDUCE THE FORMATION OF SWIMMERS.

Concentration of 2½ *M* KCl, in Sea-water, Per Cent.	Time of Exposure.	Per Cent. Swimmers Formed.
0.1		None
0.5	1½ hours	9
1.0	5 hours	16
2.0	30 minutes	16⅔
3.5	1½ hours	17¼
5.0	3 hours	33
7.5	30 minutes	11
10.0	62 minutes	11.5
15.0	10–20–31 minutes	2.5

TABLE VI.

TO COMPARE EFFECT OF TIME AND CONCENTRATION ON POLAR BODY AND SWIMMER FORMATION.

Concentration of 2½ *M* KCl in Sea-water, Per Cent.	Length of Exposure.	Per Cent. Forming Polar Bodies.		Per Cent. Forming Swimmers.
		Both.	Only First.	
3.5	1 min.	100	0	4
3.5	10 min.	57	28	3
3.5	21 min.	41.6	25	8.5
3.5	90 min.	?	?	17
5.0	1 min.	59	25	8
5.0	10 min.	18	66	6
5.0	20 min.	37	37	10
5.0	3 hr.	?	?	33
7.5	1 min.	80	13	0
7.5	5 min.	41	38	2
7.5	10 min.	50	23	6.5
7.5	20 min.	11	41	4.5
7.5	30 min.	8	39	11
7.5	45 min.	0	8	14
7.5	4 hr.	?	?	1
10	1 min.	41	41	0
10	5 min.	35	45	2
10	10 min.	12	75	1.5
10	20 min.	0	70	4
10	30 min.	4	63	7.5
10	45 min.	0	18	6.8
10	63 min.	?	?	11.5
10	3 hr.	?	?	3
15	1 min.	65	32	1
15	5½ min.	60	33	1
15	20 min.	15	65	2
15	31 min.	0	50	2.5
15	45 min.	0	9	0
15	115 min.	?	?	0

with 1 c.c. of 2½ *M* KCl + 99 c.c. of sea-water for 3–6 minutes, whereas the best percentage of swimmers,—33 per cent.,—was obtained with 5 c.c. of 2½ *M* KCl + 95 c.c. of sea-water for 3 hours. There is a decrease in maturation accompanied by an increase in differentiation as length of exposure to KCl increases. Table VI. will serve to illustrate this point. It was found that in general 3.5 per cent. of the 2½ *M* KCl in sea-water acting for 45 minutes, was most satisfactory for inducing a high percentage both of maturation and of swimmers.

In normally fertilized eggs the requirements for both phenomena, maturation and differentiation, are met at the same time by the entrance of the sperm. Why, then, in the potassium chloride eggs, should the two processes be, as it were, separated, and the optimum conditions for one so far removed from the optimum conditions for the other? It looks decidedly as if there were a distinct set of reactions for the two processes, maturation and differentiation. Whether potassium chloride shall arouse only one, or both of these must be largely a matter of the condition of the individual egg, for in some eggs both processes go on as a result of the potassium chloride treatment, while in the same culture other eggs give only one of the two reactions, or give the other very imperfectly. What those conditions in the egg are, is entirely obscure.

B. Details of Development Induced by Potassium Chloride.—F. R. Lillie has described the course of development of the potassium chloride egg, in his papers of 1902 and 1906. In giving the following account I shall be obliged to repeat many of his observations in order to make the history as clear and complete as possible, but I shall omit details largely. The eggs to be described were from two sets of experiments,—one using 7.5 parts 2½ *M* KCl to 92.5 parts sea-water for one hour (designated set *A*),—the other using 3.5 parts 2½ *M* KCl to 96.5 parts sea-water for 45 minutes (set *B*). The general behavior of the eggs in the two series is very similar, and I shall describe now one, now the other, without distinction.

The Living Egg (Set B).—The first polar body forms inside 15 minutes,—the second, when formed at all, inside 30 minutes. The eggs often form a polar lobe and appear to be attempting

cleavage. The flow of cytoplasmic material in the egg keeps up in one direction for a time, accompanied by a deepening constriction in the egg, until suddenly the direction of the flow changes, or a new wave is set up, and all appearance of constriction or cleavage is lost. Often the flow is set up in opposite directions at once, and thus the constriction plane is simulated, but one flow suddenly overcomes and obliterates the other. The polar lobes also disappear. The amœboid movements noted in so many eggs are to be seen in *Chætopterus* also, so markedly that at one time successive camera drawings showed that the egg actually moved along the slide for some little distance. Pseudo-cleavage takes place to some extent, that is, cleavage of the cytoplasm without cleavage of the nucleus. There is also indication of amitotic cleavage of nucleus and cytoplasm together. As development proceeds the nucleus increases in size, and a massing of the cytoplasmic materials in different parts of the egg can be seen. At ten or eleven hours the larvæ are beginning to swim,—several hours later than fertilized eggs. The percentage of swimmers at 20–24 hours varies greatly. Usually the larvæ were not kept longer than that, but on one occasion they were kept nearly 48 hours, and at the end of that time I find that my notes record, "Full of very active swimmers, some even having risen in the water, and all much resembling trochophores in general appearance." Many larvæ are able to swim in a comparatively straight course, others spin round and round, others do now one, now the other. There are many fusions and some fragments, any of which may be ciliated. Some are composed of a few pseudo-cells, but many are one-celled and also uninucleate. Vacuoles are noticeable in or near the surface.

Fixed Material.—Eggs from Set *B* were fixed every five minutes for the first two hours and fifteen minutes, and every half hour thereafter to 19 hours 30 minutes, with a few more at five-minute intervals during the period of chromatin distribution. Eggs from Set *A* were fixed every half hour or less.

The history of the development is in general as follows. While the eggs are still in the potassium chloride solution the first maturation division is carried through and the first polar body extruded; the nucleus of the second polar body, if formed, is

either extruded or retained within the egg, where it unites with the female pronucleus (see Figs. 1 and 2). After the eggs are transferred to normal sea-water a period of imperfect cleavage follows, which involves only the chromosomes. In the period corresponding to first cleavage, imperfect and rudimentary mitotic figures form and the chromosomes split longitudinally, but do not separate into daughter nuclei. This process is repeated in subsequent divisions and the chromosomes remain in an increasingly large, single mass, of tangled rods. This whole period is characterized by a growth of the chromatin at the expense of the cytoplasm Resting nuclei arise between successive chromosome divisions, as normally, in Set *B*, but not until several divisions have taken place in Set *A*, when a general achromatic period enters. Shortly after this general resting period in Set *A*, and perhaps a little earlier in Set *B*, a change in general behavior follows. The nucleus begins to distribute material to the cytoplasm, sometimes in the form of rods, more often in the form of granules, a certain part of which become indistinguishable in the cytoplasm as chromatin (see Figs. 3 and 4). Part of the chromatin in the cytoplasm seems to be "dissolved"; part forms small granules which are indistinguishable from the small basophile granules of the cytoplasm; part remains as scattered chromidia. The chromatin in the nuclei of some eggs also assumes new forms,—short, stout rods, granular nuclei, karyosomes, and irregular bodies of chromatin. Finally, a large part of the chromatin distributed through the cytoplasm collects in masses in various parts of the egg and forms membranes, thus giving rise to accessory nuclei (Fig. 5). The latter part of development may thus be called a period of chromatin distribution. It is during the latter period that the characteristic cytoplasmic overflow described by Lillie ('06) takes place, followed by the formation of cilia (Figs. 6 and 7). The process of chromatin distribution seems to be definitely related to that of cytoplasmic differentiation.

2. *Sodium Chloride.*—The effects of sodium chloride were similar to those of potassium chloride but not so good,—therefore, the solution was not used to any extent. Eggs in 8 c.c. of 2½ *M* NaCl + 50 c.c. of sea-water for 45 minutes, or for one hour

and 10 minutes showed some unsegmented swimmers, but those left permanently went to pieces (as also in potassium chloride permanently). Something resembling one large polar body was formed in many eggs. Cytological study was not made of these eggs, and therefore I cannot say positively whether it was a polar body or only a small extra-ovate. Morgan ('00) has said that he got no polar body formation with sodium chloride in *Chætopterus*.

B. Acids.—Six different acids were used, three mineral,—*n*/10 hydrochloric, *n*/20 sulphuric, *n*/10 nitric,—and three organic,—*n*/10 acetic, *n*/10 butyric, and *n*/20 oxalic. The results were not particularly satisfactory, and not many experiments were made. It is possible that better results might have been obtained with other concentrations or with different length of application of the acid. The results were as seen in Table VII.

Since polar body formation was not good and the appearance of the eggs was not promising, and since others had tried acids with little effect on *Chætopterus* I did not press the methods further. No normal segmented larvæ developed. As noted in the table, eggs left in 2 c.c. of *n*/10 HCl + 100 c.c. of sea-water permanently, formed a small number of swimmers. Eggs in 1 per cent. of the *n*/10 acetic acid in sea-water, for 1 or 5 minutes, formed polar bodies only, but the same solution for 10½ minutes, or 5 per cent. of the *n*/10 in sea-water, for 5 minutes, induced the formation of a few unsegmented swimmers. Of the ineffective acids oxalic was most promising, and should be tried further.

Butyric acid was used only in connection with sodium chloride and sodium hydroxide, as follows: 2.8 c.c. *n*/10 C_3H_7COOH + 50 c.c. sea-water 2 min., followed by 200 c.c. sea-water 20 min., followed by 8 c.c. of 2½ *M* NaCl + 50 c.c. sea-water 10, 20, 30, 40, 50 or 60 minutes. A very small percentage of partially differentiated eggs, non-swimming, were found next morning and one swimmer was observed in the 30-minute lot, but the hoped-for cleavage was not obtained.

The cytological effects of the acids were similar, in the swimmers, to the potassium chloride effects, but many of the eggs which did not develop far presented a very different appearance, seemingly characteristic of acid treatment. These eggs showed

TABLE VII.

n/10 Hydrochloric Acid.

Concentration.	Time.	Polar Bodies.	Swimmers.
1 c.c. + 100 c.c. of sea-water	5 min.	None	None
2 c.c. + 100 c.c. of sea-water	5 min.	None	None
2 c.c. + 100 c.c. of sea-water	permanently	None	Few
M/20 Sulphuric Acid.			
5 drops + 100 c.c. sea-water	1 hr. or permanently	None	None
1 c.c. + 99 c.c. sea-water	7 min. or 1 hr. or permanently	None	None
3 c.c. + 97 c.c. sea-water	8 min. or 1 hr. or permanently	None	None
10 c.c. + 90 c.c. sea-water	9 min. or 1 hr. or permanently	None	None
N/10 Nitric Acid.			
6 drops + 100 c.c. sea-water	1 hr.–2 hr.–permanently	None	None
1 c.c. + 99 c.c. sea-water	1 hr.–2 hr.–permenently	None	None
2 c.c. + 98 c.c. sea-water	1 hr.–2 hr.–permenently	None	None
3 c.c. + 97 c.c. sea-water	1 hr.–2 hr.–permanently	None	None
5 c.c. + 95 c.c. sea-water	5 min.–permanently	None	None
10 c.c. + 90 c.c. sea-water	5 min.–permanently	None	None
17 c.c. + 87 c.c. sea-water	5 min.–permanently	None	None
n/10 Acetic Acid.			
a 5 drops + 100 c.c. sea-water	1 hr.–2 hr.–permanently		
b 1 c.c. + 99 c.c. sea-water	1 min.–5 min.–10½ min.–30 min.–1 hr.–2 hr.–permanently.		
c 5 c.c. + 95 c.c. sea-water	1 min.–5 min.–15 min.–31 min.–1 hr.–2 hr.–permanently.		

Results:

a gave no polar bodies or swimmers.

b, 1–5 min. gave polar bodies—no swimmers.

b, 10½ min. gave polar bodies and swimmers.

c, 5 min. gave swimmers.

n/20 Oxalic Acid.			
5 drops + 100 c.c. sea-water	1 hr.–2 hr.–permanently	None	None
1 c.c. + 99 c.c. sea-water	1 hr.–2 hr.–permanently	None	None

very little, if any, nuclear growth, but in each egg several asters appeared, without spindles, and with a large, black-staining mass of chromatin (?) at the center of the aster. There was more or less breaking up to form "cells" either nucleate or a-nucleate, but no real, mitotic cleavage.

C. Alkali.—The only alkali used was sodium hydroxide, and as this was used in combination with sodium chloride it will be described under the section on the combined use of two agents.

D. Alcohol.—Very little development followed the use of

alcohol in any of the experiments. Concentrations were used of 0.5 per cent., 2.0 per cent., and 5.0 per cent., the solutions being made up with sea-water. The times varied from 3 minutes to 3 hours. One swimmer was found in eggs exposed to 5 c.c. alcohol + 95 c.c. sea-water for forty minutes. The great majority of eggs were undifferentiated. The fact that so few eggs showed any signs of differentiation makes it seem possible that this swimmer was not due to the alcohol, but to some chance sperm, since once or twice I found a swimmer in the control. This was extremely rare.

E. Potassium Cyanide.—Twenty c.c. of *n*/10 potassium cyanide + 80 c.c. of sea-water for forty minutes induced a certain proportion of eggs to form both polar bodies, other eggs only one. Moreover, the polar bodies formed *while the eggs were still in the potassium cyanide solution.* No further development followed. Since KCN is generally considered as an agent which suppresses oxidation, I tried raising the oxygen content of the sea-water after the eggs were removed from the potassium cyanide, hoping that this would induce further development, but the results were negative.

F. De-aerated Sea-water.—The results of the use of de-aerated sea-water were very similar to those induced by potassium cyanide. In both cases we supposedly have reduced oxidation rate. The sea-water was de-aerated by heating to the boiling point in a large flask for five minutes, then cooled in the same flask (rubber stoppered), immersed entirely in water. The eggs were dropped into this bottle. Some air may have re-entered during the course of the experiments. As a result of putting unfertilized eggs into this water a considerable number formed polar bodies, as many as 50 per cent. forming both polar bodies in some experiments, and 15–20 per cent. the first polar body only. The polar bodies formed rather more slowly than normally. No swimmers were formed.

G. Excess of Oxygen in the Sea-water.—Oxygen from a tank (the ordinary commercial oxygen), washed through water, was allowed to fill a wide-mouthed bottle in the bottom of which was about one centimeter depth of sea-water. The bottle was then tightly corked with a rubber stopper, and placed in the ice-chest for two or three days, and shaken a number of times during that

time. Before the experiment the bottle was brought into the laboratory. During the course of the experiment a stream of oxygen was run through the bottle. Unfertilized eggs were placed in the bottle of oxygen-saturated sea-water, and removed at various intervals, from 10 minutes to 4 hours, to dishes of ordinary sea-water on the table. The effect of the treatment was to induce the formation of a small percentage of polar bodies and swimmers. With even so short an exposure to excess of oxygen as 16 minutes, 23.7 per cent. of the eggs formed the first polar body and 2.7 per cent. formed both polar bodies,—but with an exposure to oxygen of 55 minutes 16 per cent. formed both polar bodies. A few eggs gave the appearance of one or two cleavages, but no segmented swimmers were found, and less than 1 per cent. of unsegmented swimmers.

H. Temperature Changes. 1. *Cold.*—Eggs placed in dishes of sea-water at room temperature and then removed to compartments of the ice-chest where the temperature was 9.5° C. or 12.5° C. for 15 minutes to 2¼ hours, gave a small percentage of polar body formation and also of unsegmented swimmers. The swimmers were of the ordinary type induced by KCl and described there as apparently typical of unsegmented larvæ.

2. *Heat.*—The use of heat gave by far the best results with *Chætopterus*. I consider the experiments to be described as only preliminary tests, as I did not try the method until the end of the summer, and the supply of eggs was giving out, so that I could not make complete tests by any means. By repeating the experiment a number of times, however, I was able to determine with certainty that heat will not only induce development to unsegmented swimmers in a certain percentage of eggs, but will also bring about the delicate balance of processes concerned in mitotic cleavage of a normal sort. The percentages of swimmers obtained were small, many of the cleavages were abnormal, and nearly all the larvæ became abnormal in time, but they demonstrate clearly that an agent other than the sperm can induce the segmentation of the egg of *Chætopterus*. It may be that some modification of the method, in temperature or time, will lead to the formation of entirely normal larvæ, or perhaps some added agent will be necessary. The only combination which I at-

tempted was with KCl, and that did not result favorably for the production of segmented swimmers.

The method used was to suspend small flasks of sea-water in a water bath whose temperature could be varied, and when the temperature within the flask was at, or a little above, the desired temperature, introduce the eggs. The eggs were thus suddenly subjected to a rise of temperature of several degrees. The apparatus, which was a crude one, did not keep the eggs at an exactly even temperature, and the results will be given in each case as the effect of a temperature within the limits of variation; *e. g.*, the most favorable temperature used was one which varied between 32.5° and 34.5° C. The various temperatures tried ranged from 30° C. to 47° C. From 35° C. up no swimmers were produced, although there was some possible cleavage. 30° C. induced almost no development. The eggs were left in the flask for times varying from 35 seconds to 2 hours and 9 minutes, and then removed to dishes on the table, where the temperature was about 23°–24° C. Other eggs were left in the flasks over night to cool down slowly after the end of the experiment. It is possible that in the very beginning of the experiment the temperature was lowered a little by the introduction of the small amount of water necessarily carried in with the eggs. The optimum length of exposure to the heat was 40 minutes or near it. The very brief exposures were made only for the high temperatures. Less than 25 minutes for the lower temperatures was ineffective in producing segmented swimmers, although shorter exposures than that might show some polar body formation and a small amount of cleavage.

The eggs did not usually extrude the first polar body until removed from the warm water to the cooler, but then went on promptly with one or both maturation divisions. The percentage forming both polar bodies was in general smaller than that forming the first only. In the series of which I have sections the second polar body was not seen to form at all, and only a few eggs formed the first polar body. The series of fixed eggs is not at all complete, as I expected to be able to make other series later. The sections indicate that there is sometimes formation and retention of the nucleus of the first polar body within the egg, for

eggs may be seen 24 minutes after being removed from the heat, containing two nuclei and with no polar bodies. These nuclei appear to unite in certain eggs. Other eggs may be seen with one polar body and one nucleus. There were not many eggs in which a count of the chromosomes could be made, but in one such count there appeared to be 18 chromosomes. Evidently the second polar nucleus if formed at all had united with the egg nucleus. I think, however, that the second polar spindle frequently is not formed, but that the chromosomes may go into the resting stage after the first maturation spindle, and emerge from this in the characteristic rod shape of the cleavage divisions, whether one or no polar body has been extruded.

Cleavage proceeds with more or less regularity. Unfortunately the eggs were killed at such time that only one egg was caught with the spindle of first cleavage. It seems to be in early anaphase, and although I cannot count the chromosomes exactly, owing to their twisted rod shape and the direction of the section, their number was approximately 36, which makes it seem that the number normal for cleavage, 18, is probably present in each half of the spindle. Second cleavage stages are wanting also, but eggs fixed two hours from the beginning of the experiment show the four-cell stage with spindles of third cleavage (cf. Fig. 9). In one egg in which I was able to count the chromosomes there appeared to be 18 in each cell. (As before stated, the count is not exact, and only serves to indicate whether the chromosome behavior as to numbers, in these mitoses, is proceeding approximately after the normal order.) At this time many eggs are still seen in the metaphase of the first maturation spindle. This spindle often lies in the center of the egg instead of at the periphery, but is easily distinguished from the cleavage spindle by its smaller size and by the chromosomes, which are in the maturation form. A number of eggs are unsegmented and have multipolar spindles, others are segmented abnormally. Many appear to be developing after the characteristic KCl fashion.

Later sections, between 6 and 7 hours, show many segmented blastulæ (Fig. 10). The blastulæ swim at about this time. Abnormalities are usually to be seen; some larvæ have almost no segmentation cavity, others small extra-ovates. The arrange-

ment of substances in the cells is in general normal. A good many larvæ are abnormally segmented, often one cell having stopped in an early stage of cleavage, and the others having gone on and divided further. The larvæ fixed at 21 hours (Fig. 11) are mostly abnormal, apparently, but one or two were found in the living material which showed even the long apical flagellum.

On the whole the series approaches much more nearly to the normal development than any other series I have so far been able to obtain, chiefly because here, and nowhere else, could I get mitotic cleavage to any extent. The possibility of the development of unfertilized eggs to swimming larvæ having been established, the question of inducing a cleavage process to accompany this development was the next important step. Cleavage obtained, it will now be necessary to vary conditions in such manner that the abnormalities arising in the cleavage with the present method may be avoided. The later development of the larvæ, beyond the trochophore stage, I have not attempted, as my first interest lay in the *initiation* of normal development.

IV. Combination of two Agents.

In order to form a check on the effect of the various agents upon the unfertilized eggs, artificial parthenogenesis was supplemented by fertilization in a number of cases,—sperm being added after the eggs had been treated by a physico-chemical agent. In other cases the effect of supplementing the action of one physico-chemical agent by that of another was tried, and in many instances the results of treatment with two physico-chemical agents were similar to those produced by using one such agent and fertilization.

A. Artificial Parthenogenesis Supplemented by Fertilization.—Eggs were fertilized after application of an artificially stimulating agent. The parthenogenetic agents used as a preliminary to fertilization were KCl, low temperature, and oxygen-saturated sea-water. In general it may be said that the agents, of whatever sort, which will induce development in unfertilized eggs, are prejudicial to normal development in fertilized eggs.

1. *Potassium Chloride and Sperm.*—A number of experiments were performed to test the effect of fertilizing eggs whose develop-

ment was already initiated by potassium chloride. A set of eggs was placed in 1 c.c. of 2½ *M* KCl + 50 c.c. sea-water. After 1, 3, 15 and 30 minutes eggs were removed from the KCl to sea-water. Each lot was divided, part being fertilized and part remaining in ordinary sea-water with no sperm, to form a KCl control. After 15 and 30 minutes eggs were taken from the 1 and 3 minute KCl controls just described, and fertilized. Thus material was obtained showing the effect of an increasing length of exposure to potassium chloride before fertilization, which could be compared with eggs allowed to stand various lengths of time in ordinary sea-water after a very brief exposure to potassium chloride before fertilization.

TABLE VIII.

	Time in KCl.	Time in Sea-water.		Swimmers, Per Cent.		
				Segmented.	Unsegmented.	Total.
KCl control	1 min.	0	Unfertilized	0	0	0
	1 min.	0	Fertilized	59	17	76
	1 min.	15 min.	Fertilized	20	43	63
	1 min.	30 min.	Fertilized	12.5	45	57.5
KCl control	3 min.	0	Unfertilized	0	10	10
	3 min.	0	Fertilized	38	10	48
	3 min.	15 min.	Fertilized	11	17	28
	3 min.	30 min.	Fertilized	2	10	12
	15 min.	0	Fertilized	8	4	12
KCl control	30 min.	0	Unfertilized	0	16⅔	16⅔
	30 min.	0	Fertilized	5	5	10

As may be seen in Table VIII. the eggs treated for one minute with potassium chloride, and unfertilized, produced no swimmers, whereas those of the same lot fertilized immediately after removal from the potassium chloride showed many swimmers, but of these 17 per cent. were unsegmented. The fertilized control (no KCl) fertilized normally and only two or three unsegmented swimmers were found.

In this particular experiment the record of polar bodies was not taken, but in a similar experiment,—of eggs in 1 c.c. 2½ *M* KCl + 50 c.c. sea-water for one minute,—15 per cent. formed the first polar body, and 11 per cent. formed the second also. As before, no swimmers were formed. When eggs of the same lot were fertilized immediately on removal from the KCl a large

percentage extruded both polar bodies,—and both segmented and unsegmented swimmers were produced. The production of a large proportion of the polar bodies and all of the cleavage would appear to be due to the sperm. But that the potassium chloride also had an effect, and opposed to that of the sperm, is seen in the increased percentage of *unsegmented* swimmers in the KCl + sperm material above that in the fertilized control. An exposure to potassium chloride of only one minute thus causes suppression of cleavage in KCl + sperm eggs.

A delay in sea-water before fertilizing after potassium chloride shows a slight decrease in total number of swimmers, and still further suppression of cleavage, making the percentage of unsegmented swimmers much larger, and showing a very marked decrease in segmented swimmers.

When the potassium chloride is applied for a longer time before the eggs are removed to ordinary sea-water the decrease in swimmers is much faster, differentiation to swimming forms being interfered with nearly as much as cleavage, showing a stronger potassium chloride action.

Thus it is seen that the potassium chloride initiates changes which increase with time and which are inimical to normal fertilization. They do not prevent the entrance of the sperm into the egg, but they prevent the normal behavior of the sperm in the egg. Polyspermy is induced, but the suppression of cleavage seems to refer to some other factor also, since in the sections of preserved material no more polyspermy is noted in the case of late fertilization than in early, yet the ill effects of the combination are greater with late fertilization.

Results are similar for 0.5 c.c. 2½ *M* KCl in 50 c.c. of sea-water, but they were complicated by the fact that the female did not seem to be a good one, for the fertilized control cleaved abnormally and many eggs in it did not cleave at all.

A comparison of the curves made from Table VIII. (Fig. *A*) brings out these facts graphically. *AA* represents the increase in swimmers with increased time in KCl (no sperm). *CC* represents the decrease in swimmers when the eggs are fertilized *at once* on removal from potassium chloride, but with increasing time in potassium chloride. *BD* and *B'D* break this curve up to repre-

sent the proportion of segmented and unsegmented swimmers. As is evident *C* represents 76 per cent. swimmers, of which 59 per cent., represented by *B*, were segmented, and 17 per cent., represented by *B'*, were unsegmented. The falling off in percentage of segmented swimmers with increasing time in potassium chloride is much faster than the decrease in percentage of un-

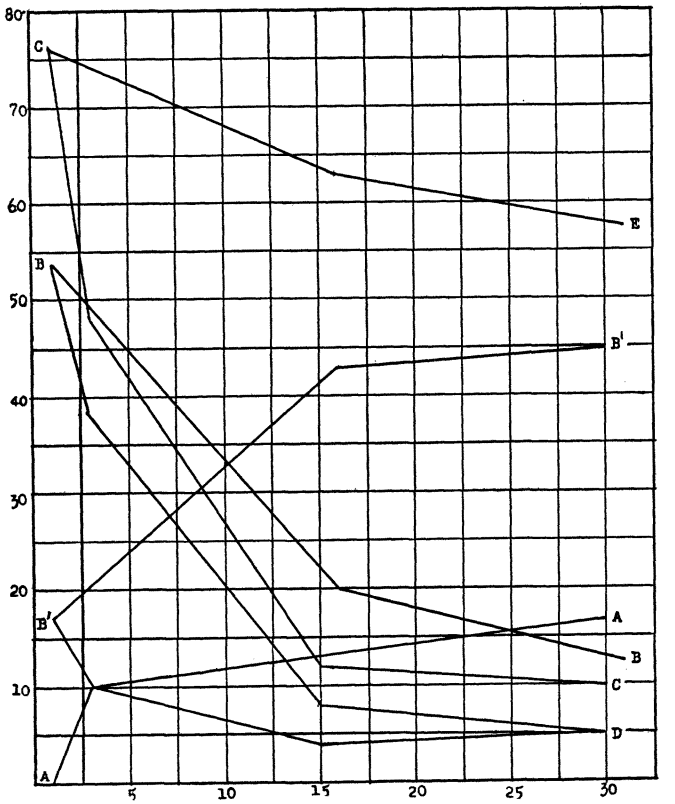

FIG. *A*. The effect of fertilizing eggs previously treated with potassium chloride. Data taken from Table VII. The abscissæ represent minutes, the ordinates the percentage of eggs developing to swimming larvæ.

segmented. Compare with these curves those showing the effect of a brief exposure to potassium chloride followed by fertilization after varying intervals in sea-water. *CE* represents the decrease in total number of swimmers. It is much less rapid than when the eggs were subjected to potassium chloride during all of the

time before fertilization (compare *CC*), but it is a decided drop. Breaking this curve up as before, to show the proportion of segmented and unsegmented swimmers,—*BB* represents the decrease in segmented swimmers, but *B'B'* shows a decided rise in proportion of unsegmented swimmers, although the total percentage of swimmers produced is less than in the fertilized control or in the lot subjected to KCl action for a brief time only (see Table VIII. fertilized after KCl 1 minute). In other words, it appears that in the eggs which have been subjected to a slight or slow KCl action the spermatozoön is unable to induce cleavage, so that we obtain many unsegmented swimmers,—while in eggs showing a greater or more rapid KCl action the changes are so great that the sperm cannot even induce differentiation, in such a doubly stimulated egg. It seems that the effect of the potassium chloride is a very decided one, which once having been established in any egg, inaugurates such changes in it that it is no longer capable of normal fertilization. (A few eggs may be able to withstand the double action.) The longer the potassium chloride acts the further the changes proceed, and the more abnormal is the larva resulting from fertilization after KCl. In short the eggs cannot endure this double stimulation any better than they can polyspermy.

These results, it seems to me, are exactly what would be expected. For if we consider the artificial stimulating agent as acting in somewhat the same way upon the egg that the sperm does, we would thus get in the egg some of the same sort of changes set up which would take place in a fertilized egg. If then we add sperm after this series is set going, the fertilization is not to be expected to be normal.

2. *Cold and Sperm.*—Eggs placed at a temperature of 9.5° C., and fertilized after removal to room temperature, showed considerable reduction in percentage of swimmers, especially segmented ones, the reduction increasing with length of time, and apparently more rapidly if the eggs were subjected to the continuous action of cold up to the time of fertilization than if an interval of time at ordinary room temperature was allowed to intervene between the application of the low temperature and the addition of the sperm. Here, as in KCl + sperm there is a ten-

dency for adverse conditions to interfere first with cleavage, and second to destroy the power of differentiation into swimmers also (see Table X.).

TABLE X.

Treatment.	Segmented Swimmers, Per Cent.	Unseg. Swimmers, Per Cent.	Total Per Cent.
Fertilized control	72.5	1.5	74
9.5° C. 30 min., then room temp. 15 min., then sperm	15	44	59
9.5° C. 1 hour, then sperm	0	12	12

3. *Excess of Oxygen in the Sea-water and Sperm.*—The effect of an excess of oxygen in the sea-water is here considered as a primary stimulus to artificial parthenogenesis and therefore included with the other experiments of fertilization supplementary to a physico-chemical parthenogenetic agent. When sperm were added to eggs which had been for varying times in oxygen-saturated sea-water, in one case the number of swimmers resulting in the experiment was reduced one half or more from that of the fertilized control. That the reduction was not due to length of time after removing the eggs and sperm from the animals was seen by making a fertilized control at each time when an experimental lot was fertilized (Table XI.).

TABLE XI.

Time in O_2 Sea-water.	Followed by Time in Sea-water before Fertilized.	Swimmers, Per Cent.		
		Segmented.	Unsegmented.	Total.
Control—0.	0.	44	22	66
16 min.	21 min.	8	25.5	33.5
Control—0.	37 min.	35.5	24	59.5
65 min.	1 min.	5	7	12
Control—0.	66 min.	37.5	27.5	65
120 min.	0.	12	5	17
Control—0.	120 min.	43	25.5	68.5

The sperm and eggs were evidently just as able to develop normally at the end of the experiment as at the beginning. Nevertheless the eggs which had been subjected to the action of an excess of oxygen showed a much lower per cent. of swimmers than the control, and the effect was greater with longer time of

oxygen action,—results entirely comparable to those in the KCl + sperm experiments.

B. Combination of Two Physico-chemical Agents.—A number of different combinations were made, using potassium chloride with other physico-chemical agents, in the hope that some other agent added to the potassium chloride would induce cleavage. The method met with little success. In fact in many cases the results were similar, in their suppression of development, to those produced by using a parthenogenetic agent and sperm.

1. *Double Potassium Chloride.*—A weak potassium chloride solution (1 c.c. 2½ *M* KCl + 49 c.c. sea-water), a concentration suited to induce maturation, was allowed to act for four minutes. The eggs were then removed to sea-water where they remained 10, 20, 40, or 60 minutes. They were then placed in a second potassium chloride solution, (5 c.c. 2½ *M* KCl + 45 c.c. sea-water),—a solution usually suited to induce the formation of swimmers. The eggs were left in this second KCl from 9 minutes to 2 hours. Almost no swimmers were formed! Some changes had apparently been initiated by the first "dose" of the potassium chloride, which prevented the second "dose" from having the effect it would have been expected to have by itself. Here, as in the combining of artificial parthenogenesis and fertilization, the time factor is important,—that is, the length of time after the initiation of development determines the extent to which development shall have proceeded, and the farther it has proceeded, that is, the longer the time, the less possibility there is of another agent inducing its normal reaction in the egg.

2. *Oxygen and Potassium Chloride.*—In the experiments with oxygen and potassium chloride, application of oxygen excess to the eggs while they were in potassium chloride made the experiment less successful than with potassium chloride alone, whereas used after potassium chloride, oxygen-saturated sea-water induced the formation of a slightly larger percentage of swimmers than the potassium chloride alone. Moreover a set of experiments in which the oxygen content of the water was raised merely by running a continuous stream of the gas through the water for a short time before, and during the experiment, had as a result the formation of a number of very normal looking blastulæ (Fig. 8),

and the next morning several swimming, apparently segmented, larvæ. The sections show a number of segmented blastulæ, but no segmented swimmers. In this case the eggs were left in 3.5 c.c. of 2½ *M* KCl + 96.5 c.c. sea-water until after the formation of the second polar body, and were then placed in the oxygen-saturated sea-water, where they were left from one half hour to four hours, approximately. Ordinary "KCl larvæ" were present in all cases, as well as the few segmented larvæ. Sections show considerable amitosis, and some pretty good di-polar spindles,—perhaps a better condition of mitotic figures, in general, than in ordinary KCl material,—and a few blastulæ, some practically normal, some abnormal. Many pseudo-blastulæ appear, *e. g.*, a large nucleated cell at one end of the larva, with a row of smaller cells at the other end separated from the large one centrally by a cavity. These smaller "cells" do not contain nuclei, but appear to have chromatin scattered in the cytoplasm. The experiment was performed on four different days, but only once did any considerable number of segmented individuals arise, unless the small, nucleated, isolated cells, appearing in one experiment, were due to cleavage followed by a falling apart of the blastomeres. I did not catch them in process of cleaving, so cannot be sure whether such cleavage was mitotic or mere fragmentation. Because of the infrequency of the result, I do not place much weight on it as a means of inducing cleavage unless in some way the results may be made more uniformly certain. However, the fact remains that oxygen-saturated sea-water used after the eggs have been subjected to the action of potassium chloride solution, appears to affect the eggs quite differently from a second "dose" of potassium chloride itself. It would appear that the oxygen has a somewhat different function to perform from that of the potassium chloride, and therefore the use of the one does not prevent the action of the other when one follows the other. But used *together* they are antagonistic.

3. *Heat and Potassium Chloride.*—As a companion result of the oxygen-saturated sea-water used after potassium chloride, may be given that obtained when heat and potassium chloride were used together. In both cases there was an increase in swimmers

above the number in potassium chloride alone. In the heat experiments the eggs were placed in sea-water, in a warm bath, the sea-water containing 2 per cent. or 7 per cent. of 2½ *M* KCl. At intervals varying from 5 minutes to 1½ hours they were removed to ordinary sea-water at room temperature. With heat alone segmented swimmers were obtained, but with the combination of the two agents, only typical "KCl" swimmers were to be found. Thus while the combination made the KCl experiment more successful, on the other hand it really interfered with the heat action.

4. *Cold and Potassium Chloride.* (Table XII.)—The action of cold combined with potassium chloride was to decrease the power of differentiation, but to increase polar body formation. 36.5 per cent. of the eggs in sea-water containing 2.5 per cent. 2½ *M* KCl at room temperature for 30 minutes, formed one or both polar bodies and 16.5 per cent. formed swimmers, unsegmented. Of eggs from the same lot, in KCl sea-water of equal strength but at a temperature of 9.5° C., 79 per cent. formed one or both polar bodies, but only 9 per cent. formed swimmers. The

TABLE XII.

Per Cent. of 2½ *M* KCl in Sea-water.	Temperature.	Time, Min.	Per Cent. Forming Polar Bodies.	Per Cent. Swimmers.
3.5	Room temp.	30	36.5	16.5
3.5	9.5° C.	30	79	9
None.	9.5° C.	30	0	0
None.	9.5° C.	30	47	0

cold alone for 30 minutes caused no noticeable development, but applied for 60 minutes it brought about the formation of the first polar body in 47 per cent. of the eggs, but no swimmers (in this particular experiment). Thus the number of eggs extruding the first polar body was as much increased, approximately, by the combination of the two treatments, as the sum of the two effects each considered alone (KCl 36.5 per cent. and cold 47 per cent., —KCl and cold together 79 per cent.). But the number of swimmers was decreased from the number induced by potassium chloride alone, although increased above that for cold alone, for in the latter case there were no swimmers produced. It would

seem that the development of the swimmers, then, was entirely due to the potassium chloride, but hindered by the low temperature. Cold alone, as noted earlier in the paper, may under favorable conditions lead to the formation of swimmers.

5. *De-aerated Sea-water and Potassium Chloride.*—Two sets of experiments were performed, to test the effect of potassium chloride in connection with de-aerated sea-water,—one set using the potassium chloride before, the other at the same time with, de-aerated sea-water. The results were not very different from those with potassium chloride alone. The percentage of eggs forming polar bodies under either treatment was about equal to that obtained with potassium chloride alone. No swimmers were formed as a result of the potassium chloride alone in this case, and none with the use of the combination, except in two cases, namely, 0.5 per cent. swimmers were formed in a lot of eggs left 1½ hours in de-aerated sea-water + 2 per cent. 2½ *M* KCl, and 3 per cent. swimmers were formed in a lot left over night in de-aerated sea-water after being in 2 per cent. 2½ *M* KCl 3 minutes. The swimmers were unsegmented.

6. *Potassium Chloride and Sea-water De-oxygenated by a Stream of Hydrogen.*—A stream of hydrogen was run through a flask or bottle of sea-water to remove the oxygen. A small amount of oxygen was doubtless present, however, entering with the eggs, etc. The eggs were placed in 3.5 per cent. of 2½ *M* KCl in sea-water, 45 minutes to one hour, where many formed either one or both polar bodies. They were then removed to the hydrogen sea-water, and remained there from 8 minutes to 3 hours, with a constant stream of hydrogen bubbling through the water. A very few looked suggestive of first or second cleavage. Next morning swimmers were found in all lots, particularly the three hour one. Some appeared abnormally segmented. Sections, however, show practically no normal cleavage, but one or two ciliated larvæ were found which were very abnormally cut up into cells. The rest were much like ordinary KCl material.

7. *Potassium Cyanide and Potassium Chloride.*—Many combinations of potassium cyanide and potassium chloride were tried. In these experiments the differentiation was in general poorer than with potassium chloride alone. A great deal of breaking

up occurred, and some possible cleavage, but probably not normal mitotic cleavage. In several of the experiments there was a suggestion in the living material of a small amount of early cleavage, but the next morning segmented swimmers were not to be found, except in one set of experiments, when one possible segmented larva appeared as a result of treatment with a solution of one drop of $n/10$ KCN in 100 c.c. sea-water for one hour, following 3.5 per cent. $2\frac{1}{2}$ M KCl in sea-water for about 45 minutes,—and another among the eggs left in the same KCN solution for $2\frac{1}{2}$ hours. Sections from this set of experiments show much breaking up into small round masses, which may be abnormal cells. The material is suggestive, but that is all. It may be that the treatment causes a slight amount of cleavage and some breaking up, but certainly rarely, if ever, like that of normal development. It is interesting to note that in a number of *Chætopterus* experiments many lots of eggs which in early stages are most suggestive of cleavage, in late stages show less differentiation than in some other experiments. It may be that unless the cleavage is normal, differentiation is more difficult with cleavage than without it.

8. *Sodium Chloride and Sodium Hydroxide.*—To 8 c.c. of $2\frac{1}{2}$ M NaCl + 50 c.c. sea-water, was added either 0.2, 0.4, or 1.0 c.c. $n/10$ $NaOH_2$. The eggs were transferred to ordinary sea-water after 20, 50, 75 and 120 minutes. Swimmers appeared in all the dishes except the 120 minute lot, and the 1 c.c. $n/10$ NaOH lot for 20 minutes or 75 minutes. The best results were obtained from the 0.2 c.c. $n/10$ NaOH lot for 50 minutes. No cleavage was noted. Something resembling a large polar body was formed, similar to that seen in the sodium chloride treatment without sodium hydroxide.

V. Discussion.

1. *Problem of the First Changes.*—The first changes in development are initiated by the entrance of the egg into sea-water from the ovary of the animal. This stage in the development consists in the break-down of the germinal vesicle, the migration of its contents to the animal pole, and the formation of the metaphase of the first maturation spindle. The freeing of the vitelline membrane from the surface of the egg, with the accompanying

formation of the peri-vitelline space, takes place at the same time. The experiments performed with reference to a determination of the factors operating to bring about these phenomena were too scattered to do more than suggest further lines of work. A few of the findings are interesting, however, in their possible bearing on the behavior of other eggs which do not go through their development in precisely the same way.

No membrane forms in *Chætopterus* as a result of fertilization, but a space arises between the egg and the vitelline membrane already present, as soon as the egg is put into sea-water. The cortical changes involved are comparable to those concerned in the formation of the fertilization membrane in Echinids, yet the development in *Chætopterus* ceases with the formation of the first maturation spindle, and all eggs, unless given some further treatment, undergo degenerative changes. The cortical changes which accompany the formation of the membrane induce only a brief period of activity, and do not lead, of themselves, to development. Loeb ('09) has said that in the sea-urchin in many cases the cytolysis which is started at membrane formation leads to the death of the egg unless checked by a second agent. It is possible that in *Chætopterus* also the death of the egg after membrane formation is due to cytolysis set up at the time of membrane formation. But it seems more probable that the changes which accompany membrane formation in *Chætopterus* represent only a certain part of those which accompany the process in Echinids, and therefore the same results do not follow in both cases.

The experiments indicate that various salts have a definite effect upon the eggs,—*e. g.*, in *n*/2 sodium chloride or *n*/2 potassium chloride the eggs form apparently normal spindles, whereas in *n*/2 calcium chloride and *n*/2 magnesium chloride they do not. Magnesium chloride induces the formation of fairly normal membranes, whereas neither potassium chloride, sodium chloride nor calcium chloride bring about this result. But neutral paraffin oil also induces normal spindles, and *Chætopterus* body fluid gives normal results in all respects, in a certain proportion of eggs. Therefore it is evident that no one specific chemical agent is necessary for this first reaction of the egg, but that it may be induced in a number of different ways. It is possible that the changes are

induced by some physical factor, such as an increase in the permeability of the egg membrane or surface caused by a change in the osmotic pressure of the surrounding medium. There is very little evidence for this so far, however.

2. *Problem of Maturation.*—In the artificial parthenogenesis of *Chætopterus* development may proceed whether one or both polar bodies have been extruded, or both have been retained within the egg. This phenomenon has been noted in other forms. Lefevre ('07) found it to be so for *Thalassema mellita*, Delage ('02) for *Asterias*, Scott ('06) for *Amphitrite*, Kostanecki ('11) for *Mactra*, Treadwell ('02) for *Podarke*, and Morgan ('00) and Lillie ('06) for *Chætopterus*.

The cytological questions of spindles and chromosomes have not been taken up in many cases. Lefevre ('07) found in *Thalassema* that when only the first polar body was cast out of the egg, the second polar spindle was to be seen deep within the cytoplasm,—as a result of which two nuclei might be formed in the egg and fuse to form the cleavage nucleus. He was unable to get an accurate chromosome count but was able to ascertain that more than the normal reduced number appeared in later stages. When no polar body was thrown off he found in most cases only one maturation division and this took place within the egg, with subsequent union of nuclei. These observations are similar to certain phenomena which I have seen in *Chætopterus*. In this egg also the second polar spindle might often be seen lying deep within the egg, and forming two nuclei which united. Counts of chromosomes in later stages indicated that at least an approximation of the normal *2n* number was present. Similarly when no polar body was thrown out two nuclei were formed which united in certain cases.

In *Amphitrite* Scott ('06) found quite a different case, apparently. Here the chromatin of the first or second polar body might be thrown off in a mass in the cytoplasm. In *Asterias* Delage ('01) observed 18 chromosomes, the *2n* number, when but one polar body had been thrown off. In *Chætopterus* Morgan ('00) found two nuclei when the second polar body had not been extruded. In *Mactra* Kostanecki ('11) observed that whether one, two, or no polar bodies had been thrown off, cleavage of

chromosomes and nuclei, unaccompanied by cell-division, occurred, followed by later cell-division with a regulation of the nucleo-plasmic relation.

There is very little in the literature relative to the physiological cause of polar body formation in particular, aside from the general question of parthenogenesis, but a few theories with regard to the retention or expulsion of the polar bodies and the consequences to the egg, may be mentioned. Scott ('06) suggests for *Amphitrite* that potassium leads to the taking up of water by the egg, that this tends to prevent the normal collapse of the egg, and thus also interferes with the formation of polar bodies. In this connection he calls attention to the fact that in *Chætopterus* maturation is hindered by strong KCl, but not by weak potassium chloride.

Delage ('01) at one time thought that the retention of the second polar body within the egg was an important factor in the parthenogenesis of the star-fish egg, "en fournissant à l'œuf les éléments qui lui manquent," but later he changed this opinion largely, his later view ('02) being that parthenogenetic development depends on the arresting of maturation at some point when the nucleus is in the mitotic phase. When the egg is reawakened, as it were, it proceeds to cleavage. In this way only is artificial parthenogenesis dependent upon polar body formation.

Loeb ('02) stated the necessity of free oxygen and OH-ions in the sea-water in certain concentration to cause or accelerate maturation in *Asterias forbesii*. He was able to prevent maturation by a lack of oxygen or by adding acid or potassium cyanide to the sea-water. The addition of sodium hydroxide or benzol was found to induce maturation. Wolfsohn ('07) observed similarly for *Acmæa* that sodium hydroxide or fat solvents brought about maturation, and that the passage of a stream of hydrogen through the water inhibited it. Also "by stopping the oxidative processes in the egg through the presence of potassium cyanide, maturation can be inhibited." In these cases, then, it would appear that maturation is bound up with oxidation processes of some sort in the egg.

In *Chætopterus* the matter is rather different. Not only is

lack of oxygen not prohibitive of maturation, but it has been determined that a solution of potassium cyanide, or the use of sea-water de-aerated by boiling, actually stimulated the eggs to form the polar bodies, and that, moreover, while the eggs were still in the solutions. In *Chætopterus*, then, may it not be that maturation is a phenomenon concerned mainly with hydrolyses? It seems to be generally agreed that hydrolyses can proceed in lack of oxygen or in presence of potassium cyanide. The action of cold used with potassium chloride is interesting in this connection. The percentage of eggs forming polar bodies in this case was greatly increased over the percentage of maturation induced by potassium chloride alone. If cold suppresses oxidative processes, this may be the reason for the increase in maturation percentage. On the other hand, eggs subjected to the action of heat do not form the polar bodies while in the warm water, and in many cases the second polar body is never extruded, even though development proceeds to the formation of swimming larvæ. Now if heat, as is generally supposed to be the case, increases the rate of all reactions, it must increase both the hydrolyses and the oxidations. Here, then, there should be a stimulus to maturation, and this is in fact seen in certain eggs which do form one or both polar bodies. But there is also either an active suppression of maturation or a lack of stimulus to it in the *majority* of eggs, for most of them form only one, or neither, of the polar bodies. Where oxidations are increased, then, there is a tendency to omit maturation. As another bit of evidence, we may include the observation that an excess of oxygen used at the same time with potassium chloride somewhat lessened the percentage of swimmers, but used *after* potassium chloride, increased it. Here again, apparently, excess of oxygen at the very beginning of development is not good. The fact that oxygen-saturated sea-water will induce a small percentage of eggs to form polar bodies, may seem contradictory. But it is possible that here we have a quite different set of reactions leading to some of the same results. For example the oxygen excess may serve to stimulate some reaction other than oxidation, only gradually bringing about an increase in the oxidation rate. Thus it may be that the reactions which are set in motion go on with a low

oxidation rate for a time, and hence are accompanied by maturation.

R. S. Lillie ('08) says of the star-fish egg, "Suppression of oxidative combined with acceleration of hydrolytic and reducing processes is indicated as a condition of the initiation process in these eggs." Loeb ('09), in commenting on Lillie's conclusion, suggests that suppression of oxidation in the egg has its beneficial effect not by allowing anaerobic processes which in themselves are of importance to the development, but by giving the egg time to recover from the injurious effects of membrane formation before proceding to further development.

Another body of observations given in the experiments should be mentioned here. With nearly all agents employed, the longer exposure to the agent or the higher concentration of it, are prejudicial to maturation. On the other hand, production of swimmers occurs in larger percentages with high concentration and long exposure. If, as suggested, the course of development is composed of two rather distinct sets of reactions, the one accompanying maturation and concerned mainly with hydrolyses, the other resulting in differentiation and requiring oxidation processes, then it seems reasonable to suppose that an agent which calls out both maturation and differentiation calls out both sets of reactions, the one gaining force a little later than the other. The two processes take place in their normal relation when a certain concentration and time is used, but when this concentration or time is increased, the normal time relation between the two is interfered with and the second reaction arises before the first is completed. That is, the hydrolyses become obscured by the oxidative processes which always accompany differentiation. If these oxidative processes be of high enough rate to start up the processes that lead to differentiation more rapidly than the hydrolyses are forwarding the processes leading to maturation, then obviously the later developmental phenomena may come in and cut short the maturation phenomena.[1]

[1] This actually seems to be the case when the second maturation spindle is apparently drawn down toward the center of the egg (as the female pronucleus would be, normally) before mitosis has been completed and the second polar body thrown off.

A second possibility to account for the effect of the potassium cyanide and the de-aerated sea-water has been suggested, namely, that these agents act as stimulants which are followed by a depressing effect, one acting directly, the other through allowing an accumulation of carbon dioxide. According to this hypothesis potassium cyanide may be regarded as a protoplasmic poison which first stimulates, then depresses. It is to be remembered in this connection that no development to swimmers follows maturation brought about by potassium cyanide or by de-aerated sea-water. The de-aerated sea-water under this theory may be thought to act indirectly, by permitting the accumulation of carbon dioxide given off by the egg as a result of the intracellular oxidations. No more carbon dioxide would be given off supposedly, than under normal conditions, but in this case there is no oxygen to keep the balance of processes normal. The accumulation of carbon dioxide might then act as a stimulus, as it is known to do in other cases. Carbon dioxide above a certain amount, however, is toxic to an organism, and after it has reached this amount with the eggs it may act as a narcotic, inhibiting further development. A simple experiment to test this might easily be performed with a stream of carbon dioxide. Thus the potassium cyanide and carbon dioxide would, according to this hypothesis, act first as stimulants, and then as depressants. Just what physiological processes are involved in such "stimulation" is unknown.

3. *Problem of Differentiation.*—The term differentiation is used here to denote the developmental changes which occur after maturation and which lead, when complete, to the production of swimmers. Cleavage may be omitted in the process of differentiation. Cleavage is, of course, essential to the production of *normal* larvæ, but certain differentiations may go on without it, even to the production of swimming larvæ.

Differentiation may be induced in *Chætopterus* by a varied list of agents, both physical and chemical. Nearly every agent tried induced either differentiation or maturation or both, and it is probable that the list of possibilities is very extensive indeed. Besides the agents which I have mentioned in this paper, Loeb ('01) has brought about development in *Chætopterus* by the use

of magnesium chloride, calcium chloride, cane sugar, potassium bromide, potassium nitrate and potassium sulphate. The egg must be in a very labile state, such that almost any slight impetus will force it from its condition of unstable equilibrium.

There are, however, indications of specific needs of the egg, satisfied more or less completely by the various methods of treatment. One of the most obvious needs, it seems to me, is for oxygen. In this respect, as before stated, there shows up a distinct difference between the maturation and differentiation processes. Although maturation is called out in lack of oxygen or in low rate of oxidation, differentiation, at least to the point of forming swimmers, is often interfered with by these conditions. A brief suppression of oxidation by potassium cyanide, following potassium chloride interfered with the production of swimmers in *Chætopterus*. On the other hand, increased supply of oxygen in the sea-water after treatment with potassium chloride led to some of the best results obtained. By far the most normal results were obtained from the application of heat for a stated length of time. Although heat may act in many ways, it is very probable that one of its effects is to increase the rate of oxidations within the egg. It is also well-known that heat increases the permeability of certain membranes to oxygen. It may be, therefore, that heat is an important aid to the oxidations necessary to differentiation. Greater oxygen pressure in the sea-water surrounding the egg will supply the need to a certain extent, but not so surely as will heat.

In plant seeds it has been found by Crocker ('06) in several cases, that the factor which causes the dormant period between the formation of the embryo and the germination of the seed is an insufficiency of oxygen in the seed due to the semi-permeability of a membrane of the seed to oxygen. When greater oxygen pressure was supplied the delayed germination proceeded. In one case heat increased the permeability of the membrane to oxygen and brought about the same result as did increased oxygen pressure in the environment of the seed. The seed in its dormant condition is of course different from the unfertilized egg in that the embryo is already formed, but the conditions that determine dormancy and the awakening from dormancy may

not be very different in the two cases. In the case of several animals the same agents which will arouse a dormant seed will arouse a dormant egg,—that is, acids, bases, heat, increased oxygen pressure, etc. The effect of these agents in a number of seeds has been traced by Crocker and others, to the effect of the agents in making the seed membranes more permeable to water or to oxygen, according as the need might be. It would be going too far to suggest that the egg is always dormant for the same simple reason, an impermeability of its membrane to water or to oxygen,—but certainly some physical or chemical change is necessary, which may be induced either by the sperm or by some non-living physical or chemical agent,—the problem being to find just the need of the egg, that is, what is lacking to cause it to continue its development. In the case of *Chætopterus* one of the factors lacking for differentiation would seem to be sufficient oxygen or sufficiently high rate of oxidation.

The question of the semi-permeability of the plasma-membrane seems to be a very vital one. R. S. Lillie ('11), in his study of artificial parthenogenesis in *Arbacia*, states that the critical change in the egg to which the initiation of cleavage is due is a well-marked and rapid increase of the permeability of the plasma-membrane. He has lined up a number of salts whose power to induce development runs parallel to their power to increase membrane permeability. Among these salts are the chlorides, bromides, and nitrates. The potassium salts of all of these, and the sodium, calcium, and magnesium salt of the chloride, have been found to induce development in *Chætopterus*, and it seems probable that they may exert the same sort of effect on this egg that they do on *Arbacia*. Lillie ties up the change in permeability with a change of electric polarization ('12), and a sudden diminution of the resistance to the progress of the oxidative energy-yielding reaction, brought about by allowing the escape of the products of oxidation,—particularly carbon dioxide ('09).

It is a well-known fact that developing eggs need much more oxygen than resting, undeveloping ones. Various theories have been brought up to account for this need. Loeb ('09) considers that certain oxidative processes are advantageous in checking the cytolysis set up in the initiation of development,—which

cytolysis goes on to the destruction of the egg unless checked. Child ('11) suggests that the egg is a cell overloaded with food-stuffs and structural obstacles to metabolism,—that therefore increased metabolism is necessary for its further development. To quote him more exactly, "Physiologically the gametes are in the extreme stages of senescence and can be saved from death only by some regulatory change, which permits increased metabolism, and more specifically probably increased oxidations and syntheses."

All the facts seem to point to the conclusion that the reactions concerned in maturation and those in differentiation are somewhat different. F. R. Lillie ('11) has shown for *Nereis* that there may be a separation of the stimuli to the two processes even in the case of fertilization,—maturation being induced by contact of the sperm with the egg,—whereas differentiation does not follow except with penetration of the sperm within the egg. Loeb ('09) has noted in *Asterias forbesii* that the agent which causes development may hinder maturation, and has suggested that in eggs in which the entrance of the sperm calls out both maturation and development, the same chemical agent may call out both processes. This proves true for *Chætopterus* within certain limits, as already noted, but at the same time the evidence points to the presence of two different sets of reactions for the two processes.

4. *Problem of Cleavage.*—Inasmuch as most agents call out differentiation without cleavage in *Chætopterus*, and only two so far have induced the formation of segmented larvæ, there is the possibility of studying some of the fundamental questions of cell-division here.

Most writers on cell-division have attacked it mainly from the morphological side, making it a question of asters and centrosomes. Cleavage, according to Boveri, is a matter of the presence of the centrosome and of its normal activity. In *Chætopterus* centrosomes may be present, but they do not lead to cleavage necessarily. They may divide to form a group of centrosomes in the astrosphere, or they may not be visible at all in the aster. The mitotic figure is extremely abnormal in the KCl material, and cleavage is absent. There is certainly a con-

nection between the two facts of abnormal mitotic figure and failure of cleavage, whether one or the other is causal, or whether both are caused by the same thing and merely accompany each other, as many think.

It is certain also that the presence of the normal number of chromosomes in the cleavage nucleus does not insure cleavage. In many cases where only one polar body is extruded the *2n* number of chromosomes is present, but cleavage does not follow. In the heat experiments, which show the best results that I have obtained, only one polar body, or none, was thrown out, and the *2n* number appears to be present therefore. Here cleavage was obtained.

According to R. S. Lillie ('11) the mitotic figure represents an electric field of force, formed because of the more or less sudden increase in permeability at two points on the egg surface, to ions of the opposite sign from those already determining the sign of the egg protoplasm. In his experiments with *Arbacia* eggs he found that it was necessary not only to increase the permeability of the membrane in order to induce cleavage, but also to increase it so that the inflow might take place at a certain rate. If this interpretation of cleavage be correct, then it may be that in the case of *Chætopterus* the agents which cause differentiation without cleavage are such as to cause increased permeability, but not with sufficient rapidity to lead to cleavage. Heat, on the other hand, may act by causing more sudden increase.

If, on the contrary, cleavage be thought of as conditioned, not by electrical phenomena, but by a certain viscosity of the egg, as suggested by Loeb ('92) when he says that the checking of cell-division in a hypertonic solution may be due to a raising of the viscosity of the protoplasm as a result of loss of water, then it seems possible that KCl and other agents which are thought to extract water from the egg, extract it to so great a degree that the egg is unable to form the cleavage plates because of too great viscosity.

Normal cleavage requires that certain processes shall take place in correlation. It was evident from the KCl material that reactions were taking place in the egg which determined

chromosomal cleavage, and cytoplasmic cleavage. But the two were not correlated. The two processes did not occur at the same time, nor in any morphological relation to one another. The problem of inducing both processes to go on at the same rate and in morphological correlation is met by the heat treatment. It seems very possible that the question of permeability is concerned not only with the external egg membranes, but that a concentric system of semi-permeable membranes or regions, involving, perhaps, alveolar and nuclear membranes, are important factors. F. R. Lillie ('08) has shown definitely that the ground substance of the eggs of *Chætopterus* consists of four concentric layers, differing from one another in density and in aggregation of characteristic granules. If these regions and the various membranes must all become permeable and in such manner that entrance and exit of substances can take place at a certain rate, in order that reactions which should be correlated may be set up at approximately the same time, it seems very probable that the power of heat in inducing the most normal results so far obtained with *Chætopterus* is due in part to the fact that its effect is very quickly felt throughout the egg. (Other agents doubtless permeate more slowly.) In this way regions and membranes deep within the egg may be affected at approximately the same time as the more superficial ones, and thus the reactions which arise, may arise in correlation, and the nucleoplasmic relations normal for development may be set up in the egg. F. R. Lillie ('12) has concluded from his studies on the effects of partial fertilization in *Nereis* that "the establishment of normal metabolic interchange between the nucleus and the cytoplasm must be regarded as a fundamental function in artificial parthenogenesis." Any agent, then, which will affect the inner regions of the egg at the same time as the outer, may be expected to have a great advantage over one which is slow to penetrate—in that it has a much better chance of establishing normal correlations and therefore normal metabolic interchange.

5. *Artificial Parthenogenesis Supplemented by Fertilization.*—Loeb ('07) states that sea-urchin eggs fertilized after treatment with hypertonic sea-water do not develop normally, but often show multipolar spindles.

Herbst ('07) found that when he fertilized eggs of *Sphærechinus* after subjecting them to the action of butyric acid he got polyspermy and abnormal larvæ, but that this effect followed only when a certain amount of time was allowed to elapse after the application of the acid, before fertilization. With *Chætopterus* it may be said that in general any agent which induced artificial parthenogenesis interfered with normal development when fertilization was used after the artificial parthenogenetic agent. The injurious effects were greater, usually, with increased time allowed for the action of the parthenogenetic agent before fertilization, but very brief action also interfered with normal fertilization. It seems to me that these facts furnish evidence that the development induced by an artificial parthenogenetic agent is similar in its working to that induced by a spermatozoan. If the primary effect of fertilization or of artificial stimulation is regarded either as an increase in the permeability of the egg, or a starting up of cytolysis, it seems reasonable to suppose that the use of the two agents in the same egg should increase the permeability to an excessive degree or cause too rapid or extensive cytolysis, and therefore produce abnormal results. The time factor also adds its effect, in that the farther development has proceeded before the application of a second agent suited to initiate development, the more difficult it is for such an agent to produce its normal effect.

6. *Combination of Two Physico-chemical Agents.*—When we consider the combination of two physico-chemical agents we have a somewhat different proposition, although essentially the same. In this case the physico-chemical agents may or may not duplicate each other in reaction, according to their nature. If they do act as duplicates their combination appears to lead to injurious results, as in the case of adding sperm to artificial parthenogenesis. For instance when one solution of potassium chloride was followed after an interval by another solution of potassium chloride, practically no development followed. Maturation was initiated as a result of the first treatment, thus showing that the solution was effective, but when a second "dose" was applied it was unable to produce its usual effect,—indicating that the reaction had already proceeded to a point where a

stimulus of the same sort as that which initiated the development could no longer be effective. Duplicating the first stimulus would do no good. The time for the effective application of potassium chloride had passed. A stimulus of a different sort was needed.

When the action of the one agent supplements that of the other, however, the success of the experiment is increased. For example, potassium chloride added to heat reduced the percentage of segmented swimmers below the record for heat alone, but it increased the percentage of unsegmented swimmers above that for potassium chloride alone. As will be recalled, potassium chloride alone does not induce cleavage, but heat does do so. In this case, then, it is evident that heat is the better agent, since it induces a more normal result. It seems probable that the reactions brought about by the potassium chloride are incomplete in some way. Therefore when the two are applied together the effect of the potassium chloride is heightened, because some of the incomplete reactions are completed or supplied by the heat, whereas the heat effect is lessened because its working is fairly complete in itself, and is only injured by duplicating.

Summary of Discussion.

It is quite impossible in the present state of uncertain knowledge with regard to physiological causes and effects, as well as in view of the small number of the experiments brought forward in this paper, to draw any definitive conclusions as to the causes of the initiation of development in *Chætopterus*. I should like merely to summarize certain suggestions which seem to me most in line with the findings.

1. The great variety of agents which will induce the development of *Chætopterus* eggs indicates that the egg is in a very labile state of equilibrium, and that the same results may be reached by different processes.

2. Development may be resolved into a separable series of steps. A graded series of stimuli can be determined by which development may be made to stop at almost any step in the process.

3. The cortical changes which accompany membrane formation

in *Chætopterus* are associated with a very brief period of activity and are not sufficient of themselves to induce development.

4. Development to swimming larvæ may take place whether one or both polar bodies are extruded, or if both are retained within the egg.

5. Maturation and differentiation may be controlled by different conditions, indicating more or less distinct sets of reactions for the two processes.

6. Maturation may go on in reduced oxygen supply, or in suppression of oxidation by potassium cyanide. Hydrolyses are probably concerned in maturation, therefore, or other processes which can take place with very low rate of oxidation.

7. High rate of oxidation interferes with maturation.

8. Differentiation requires an increased supply of oxygen over that contained in the resting, unfertilized egg.

9. Differentiation involves oxidation. Suppression of oxidation by potassium cyanide, even for a brief time, during the differentiation period, hinders differentiation.

10. Excess of oxygen in the sea-water after the completion of maturation may induce cleavage.

11. The effect of heat in inducing cleavage is probably due in part to the intake of more oxygen by the egg, owing to increased permeability of the membrane, also to the increase in rate of oxidations.

12. Cleavage requires that the normal nucleo-plasmic relations for development shall be set up, such that reactions may take place in correlation. This is attained by the action of heat which affects all regions and membranes of the egg at approximately the same time.

13. The use of an artificial parthenogenetic agent before fertilization is prejudicial to normal development.

14. The use of two physico-chemical agents to induce artificial parthenogenesis suppresses the development called out by either, unless the action of one is supplementary to that of the other.

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Explanation of Plate I.

All figures were drawn with camera lucida with Leitz apochromat 2 mm. oil immersion objective, and Zeiss No. 6 compensating ocular, except Fig. 1 for which compensating ocular No. 12 was used.

Fig. 1. Egg fixed after remaining 30 minutes in 7.5 c.c. of 2½ *M* KCl + 92.5 c.c. of sea-water. The second maturation spindle is seen lying deep within the cytoplasm of the egg. Two chromosomal vesicles are shown at either end of the spindle. (It is entirely normal for these vesicles to arise in the formation of the nuclei after division.)

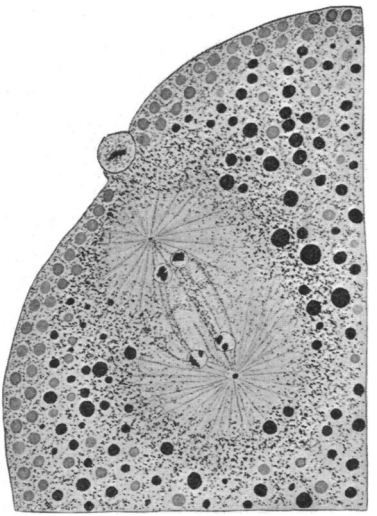
Fig. 2. Egg fixed after 35 minutes in 3.5 c.c. 2½ *M* KCl + 96.5 c.c. sea-water. The second polar nucleus and female pronucleus are about to unite to form the cleavage nucleus. The first polar body was seen in another section.

Fig. 3. Egg remained in 7.5 c.c. 2½ *M* KCl + 92.5 c.c. sea-water 60 minutes. Fixed five hours after the beginning of the experiment. Rods and granules of chromatin are seen migrating out from the nucleus into the cytoplasm.

Fig. 4. Egg treated as described for Fig. 3. Rods of chromatin previously cast out of the nucleus are seen lying in the cytoplasm. The nucleus is in the achromatic state.

Fig. 5. Egg remained in 7.5 c.c. 2½ *M* KCl + 92.5 c.c. sea-water 60 minutes. Fixed 14 hours after the beginning of the experiment. The figure represents an egg in the process of becoming multi-nucleate. Nuclei appear to be "condensing" from a large mass of scattered granules. Two polar bodies are shown.

Fig. 6. Multi-nucleate, unicellular, swimming larva. Egg treated with 3.5 c.c. 2½ *M* KCl + 96.5 c.c. sea-water 45 minutes. Fixed 14 hours and 15 minutes after the beginning of the experiment. A circle of nuclei surround a mass of yolk material and cytoplasmic granules.



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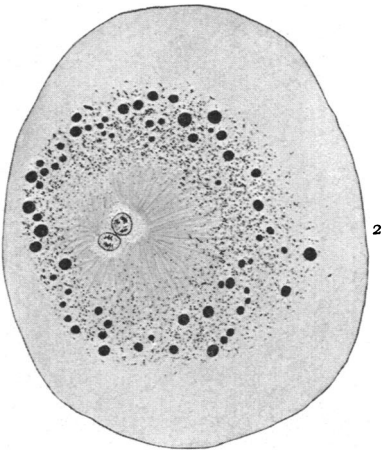

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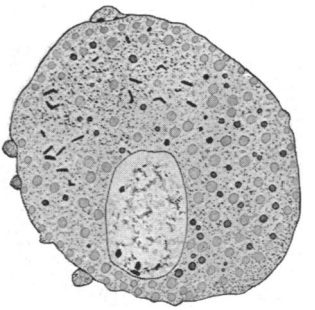

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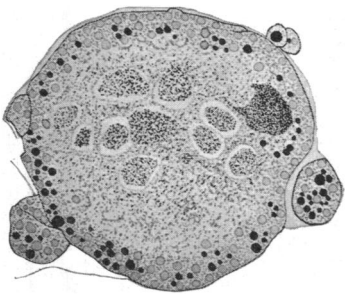

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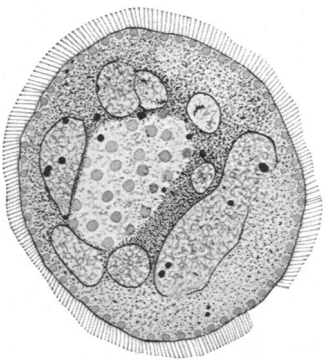

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Explanation of Plate II.

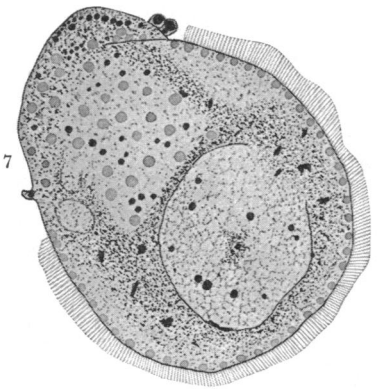
Fig. 7. Uninucleate, unicellular, swimming larva. Treated as described for Fig. 6. One or two very small nuclei and several ragged masses of chromatin are seen outside the main nucleus. The yolk mass is chiefly in the half of the egg nearer the animal pole. Two polar bodies have been thrown out.

Fig. 8. Blastula. Egg treated with 3.5 c.c. 2½ *M* KCl + 96.5 c.c. sea-water until the formation of the second polar body, then with sea-water through which a constant current of oxygen was passing. Fixed 4 hours after the beginning of the experiment.

Fig. 9. Four cells of a six-celled specimen which had been subjected to sea-water heated to 32.5° C.–34.5° C. for 40 minutes. Fixed 3 hours and 15 minutes after the beginning of the experiment.

Fig. 10. Blastula. Egg treated as described for Fig. 9. Fixed 7 hours and 5 minutes after the beginning of the experiment.

Fig. 11. Larva. Egg subjected to sea-water heated to 33° C.–34.2° C. for 42 minutes. Fixed 21 hours and 16 minutes after the beginning of the experiment. Other larvæ of similar appearance were ciliated.



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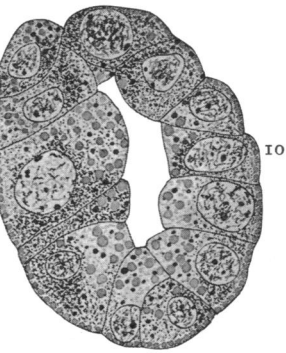

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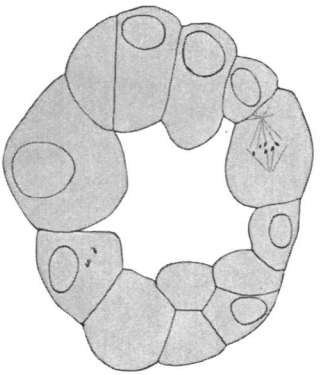

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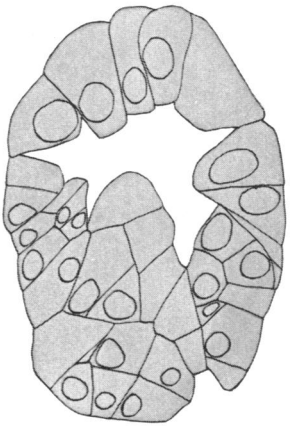

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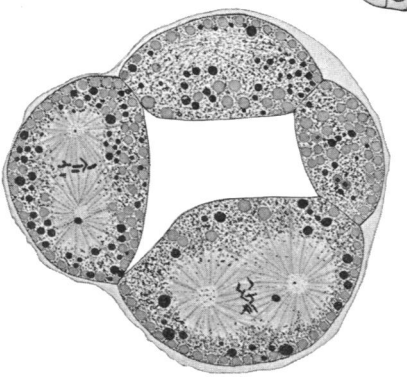

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